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Recovery and indexing of avocado plants (*Persea americana*) from embryogenic nucellar cultures of an avocado sunblotch viroid-infected tree

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Abstract

Avocado (*Persea americana* Mill.) plants were recovered from somatic embryos derived from the nucellus of an avocado sunblotch viroid (ASBVd)-infected tree ('Vero Beach' SE2). Embryogenic cultures were induced on semi solid medium consisting of B5 major salts, MS minor salts and organics, 45 g sucrose and 0.41 μM picloram, and were maintained as suspension cultures in MS3:1N medium (MS containing 12 mg l^{-1} NH_4NO_3, 30.3 mg l^{-1} KNO_3, 0.41 μM picloram and (in mg l^{-1}) thiamine HCl, (0.4), myo-inositol (100), and sucrose (45,000). Somatic embryos developed on semi solid MS medium without growth regulators. Plants were recovered from mature somatic embryos on semi solid MS supplemented with 4.44 μM BA and 2.89 μM GA_3, and thereafter were maintained on semi solid MS basal medium. RT-PCR indexing of embryogenic cultures, somatic embryos and leaf tissue of regenerants demonstrated that all developmental stages were ASBVd-positive. Variation in the viroid increased with developmental stage. Five ASBVd variants were isolated from embryogenic cultures, five ASBVd variants were isolated from somatic embryos and 33 ASBVd variants were isolated from leaves of regenerated plants. Nucellar culture cannot be considered a strategy for elimination of ASBVd from infected elite avocado material, and appears to stimulate variation in the viroid.

Abbreviations: ASBVd – avocado sunblotch viroid; NPT – nucellar plant; PEM – polyembryonic mass; RT-PCR – reverse transcription-polymerase chain reaction; SE – somatic embryo

Introduction

Avocado sunblotch viroid (ASBVd) is the causal agent of the avocado sunblotch syndrome, an important disease of avocado (*Persea americana* Mill.). Although asymptomatic trees can occur, the most common symptoms are depressed yellow and reddish areas on the fruit, leaf-bleaching associated with vascular tissue, leaf variegation, scaly bark, whipped branches and decreased tree health (Dale et al., 1982). The viroid is a circular, single-stranded RNA molecule of 247 nucleotides in the *Avsunviroidae* family, a group of viroids that self-cleave *in vitro* through hammerhead ribozymes (Palukaitis, 1979; Symons, 1981; Daros et al., 1994). ASBVd is transmitted via seed, vegetative material, pollen, mechanical tools, and possibly root grafting (Desjardins et al., 1979). Sequence variability of viroids affecting a single host has been associated with variability of symptom expression in ASBVd infected plants (Schnell et al., 2001a; Semancik and Szychowsky, 1994).

Viroids in the *Avsunviroidae* family replicate in the chloroplasts (Flores et al., 1998; Navarro et al., 1999); whereas, viroids of the *Pospiviroidae* family, e.g., citrus exocortis viroid, replicate in the nucleus and can be eliminated from infected clonal material by micrografting (Navarro et al., 1975) and nucellar culture (Bitters et al., 1972). Suarez et al. (2005) demonstrated that micrografting does not eliminate ASBVd from infected avocado plants. The original objective of the current study was to determine if ASBVd-free avocado plants could be recovered from embryogenic cultures derived from the nucellus.

Materials and methods

Induction and maintenance of embryogenic cultures

Embryogenic cultures were induced from the nucellus of immature avocado seeds according to existing protocols (Witjaksono et al., 1999). Small fruits (0.5 mm diam.) of ASBVd-infected 'Vero Beach' SE2 were collected from the National Germplasm Repository of the USDA ARS Subtropical Horticultural Research Station in Miami, FL. Fruits were washed and surface-disinfested in a 1.25% (v/v) sodium hypochlorite solution with five drops of Tween 20® per liter, rinsed three times with sterile deionized water and bisected under aseptic conditions. The zygotic embryo and endosperm were removed from each seed and discarded, and the nucellus was inoculated onto induction medium (Witjaksono and Litz, 1999a). Induction medium consisted of B5 major salts (Gamborg et al., 1968) supplemented with MS (Murashige and Skoog, 1962) minor salts, 0.41 μM picloram and (in mg l^{-1}) thiamine HCl (0.4), myo-inositol (100), sucrose (30,000) and 8 g l^{-1} TC agar (Carolina Biological Supply). Sterile medium was dispensed in 10 ml aliquots into sterile disposable Petri dishes (60 × 15 mm). There were four explants per Petri dish (81 Petri dishes). Petri dishes were maintained in darkness at 25°C. The number and percentage of embryogenic cultures were recorded weekly for 10 weeks after induction. Embryogenic cultures were also induced from zygotic embryos of ASBVd-free 'Hass' (20 Petri dishes) as the ASBVd-free control and from ASBVd-infected 'Irwin' (77 Petri dishes) and 'Yama' (82 Petri dishes) as ASBVd-positive controls. 'Lima Late' and Vero Beach'1 embryogenic cultures had been induced previously from zygotic embryos and were additional ASBVd-positive controls.

Embryogenic cultures were transferred to sterile semi solid maintenance MSP medium (Witjaksono and Litz, 1999a) in 60 × 15 mm Petri dishes and incubated in darkness at 25°C. MSP medium was similar to induction medium, but instead of B5 major salts it contained MS major and minor salts with 8 g l^{-1} TC agar. Cultures were transferred to medium of the same formulation at monthly intervals for 3 months. Approximately 200 mg of 15-day-old embryogenic cultures from MSP were inoculated into 125 ml flasks containing 40 ml of liquid MS3:1N, which consisted of MS medium modified to contain 12 mg l^{-1} NH_4NO_3 and 30.3 mg l^{-1} KNO_3, 0.41 μM picloram and (in mg l^{-1}) thiamine HCl, (0.4), myo-inositol (100), and sucrose (45,000). Flasks were sealed with heavy-duty aluminum foil, secured with Parafilm® and maintained in semi darkness at 25 °C on a rotary shaker at 120 rpm. Cultures were transferred biweekly to fresh medium of the same formulation.

Somatic embryo development

Embryogenic cultures were sieved through sterile 1.8 mm mesh nylon filtration fabric; the smallest fraction was air-dried on several layers of sterile Kimwipes® to remove excess liquid medium and finally inoculated onto 20 ml semi solid somatic embryo development (SED) medium in sterile Petri dishes (100 × 20 mm) (Witjaksono and Litz, 1999b). SED consisted of MS medium with (in mg l^{-1}) thiamine HCl, (0.4), myo-inositol (100), sucrose (30,000) and 6 g l^{-1} Gel-Gro gellan gum (ICN Biochemical). Approximately 100 mg embryogenic culture was evenly spread over the medium surface of each Petri dish and incubated for 8 weeks in darkness at 25 °C. Fifty Petri dishes for each genotype were evaluated 8 weeks after inoculation. The total number of somatic embryos was determined, i.e., the number of opaque and hyperhydric somatic embryos per Petri dish. Data were analyzed by ANOVA and means were separated by the *t*-test.

Opaque-white somatic embryos (>0.5 cm diam) were inoculated onto somatic embryo germination medium (SEG) (Witjaksono and Litz,

1999b). SEG consisted of MS medium supplemented with 4.44 μM BA and 2.89 μM GA_3 and (in mg l^{-1}) thiamine HCl (4), myo-inositol (100), sucrose (30,000) and Gel Gro gellan gum (3,000). The medium was autoclaved and dispensed in 25 ml aliquots into 100 × 20 mm Petri dishes. Seven white-opaque somatic embryos were inoculated into each Petri dish (75 Petri dihes). The plates were sealed with Parafilm® and were stored in translucent plastic boxes in a 16-h photoperiod provided by cool white fluorescent tubes (40–50 μmol s^{-1} m^{-2}) at 25°C until roots and/or shoots were visible. Plants were recovered from white-opaque somatic embryos 3–4 months after inoculation onto SEG, and were maintained on minimal medium.

Medium sterilization

The pH of all media was adjusted to 5.7–5.8 prior to addition of gelling agent. Media were sterilized by autoclaving at 121°C at 1.1 kg cm^{-2} for 15 min.

RT-PCR Indexing

Embryogenic cultures, somatic embryos and regenerated plants of 'Vero Beach' SE2 were indexed for ASBVd by RT-PCR (Schnell et al., 1997). Embryogenic cultures and somatic embryos were indexed 12 months after induction; whereas, regenerated plants were indexed 6 months later. Tissue consisted of four samples of embryogenic cultures 14 days after the last subculture in MSP medium, four samples of somatic embryos (≥5 mm diam) on SED medium and four samples from leaves of each of the regenerated plants. Embryogenic cultures of 'Hass' 11.4.3 were used as the negative control and floral tissues of 'Vero Beach' SE2 and embryogenic cultures of 'Irwin', 'Lima Late' and 'Yama' were used as the positive controls. RNA was extracted from approx. 100 mg of each type of tissue (Schnell et al. 1997).

First strand cDNA was synthesized using 10 μl of isolated RNA, 3 μl 5× first strand buffer (50 mM Tris HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 0.1 M DTT) and 0.5 μg forward primer (5′-AAGTCGAAACTCAGAGTCGG-3,) complementary to nucleotides 68–87 of ASBVd variant J02020 in the upper conserved region (Bar-Joseph et al., 1985). The mixture was incubated at 100°C for 5 min, placed on ice for 2 min and allowed to stand at room temperature for 1 h. Ten microlitre of a cDNA reaction mixture containing 2 μl of 5× first strand buffer, 25 mM of each dNTP (ATP, CTP, GTP, TTP), 500 μM β-mercaptoethanol, 20 units of RNAsin (Promega), and 200 units M-MLV Reverse Transcriptase (Gibco BRL) was added to each annealing reaction and adjusted to a total volume of 25 μl with sterile water. The reactions were incubated for 150 min in a stationary water bath at 42°C.

First strand cDNA fragments were amplified using 5′-end labeled (6-FAM) forward primer (5′-AAGTCGAAACTCAGAGTCGG-3′ complementary to ASBVd J02020 nucleotides 68–87) and 5′-end labeled (HEX) reverse primer (5′-GTGAG AGAAGGAGGAGT-3; homologous to ASBVd variant J02020 nucleotides 88–104) in a 25 μl reaction mixture containing 2.5 μl of first strand cDNA mixture reaction and 22.5 μl of a mixture of 10× PCR buffer [10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin], 25 mM of each dNTP, 1.25 units of Amplitaq DNA Polymerase (Perkin Elmer) and 10 μM of each primer. The amplifications were performed in a DNA Thermal Cycler PTC 100 (MJ Research) for 40 cycles under the following conditions: 30 s at 94°C, 30 s at 55°C and 30 s at 72°C, with a final extension of 7 min at 72°C. Amplified cDNA products were separated by gel electrophoresis in 1% molecular grade agarose (Sigma) in 0.5× TBE and visualized with ethidium bromide.

Cloning, sequencing and variant analysis

cDNA amplified fragments were cloned using TOPO® Cloning (Invitrogen) and the recovered *E. coli* colonies were cultured overnight in 100 μl of SOC broth with 100 mg l^{-1} ampicillin on a rotary shaker at 200 rpm and 37°C. The bacterial cultures were pelleted and thereafter re-suspended in 50 μl 10 mM Tris-HCl (pH 8.0); this was used as the template for PCR insert amplification. Cloned inserts were amplified using M13 forward and reverse primers (Promega) in a PCR reaction mix containing 10X buffer with 15 mM $MgCl_2$, 2.5 mM of each dNTPs, 10 μM of each primer, 0.3 units of Amplitaq DNA Polymerase and the volume was adjusted with 9.24 μl of sterile filtered DI water. The amplifications were performed in a DNA Thermal Cycler PTC100 for 30 cycles under the following conditions: 30 s at

94°C, 30 s at 60°C and 2 min at 72°C, with a final extension of 7 min at 72°C. Amplified inserts were sequenced using the ABI Prism Dye Terminator Cycle and products were analyzed in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) using ABI Prism DNA sequencing analysis software. The sequence data were manually aligned using Sequencer 4.1 (Gene Codes) and compared among them and against previously reported ASBVd variants.

Data analysis

IML and cluster analysis (Ward's minimum variance method) were used to group the ASBVd variants (SAS, 2004).

Results

Plant regeneration from nucellar cultures

Only four embryogenic cultures were induced from the 164 explanted 'Vero Beach' SE2 nucelli after 10 weeks. Embryogenic cultures (PEMs) were transferred to MS3:1N, where they rapidly proliferated in suspension, and somatic embryos developed following transfer of embryogenic cultures onto SED medium. After 8 weeks on SED medium, there were an average of 12.06 white-opaque and hyperhydric somatic embryos (> 0.5 cm diam.) per Petri dish, of which approx. 56.4% were white-opaque. Plants were regenerated from white-opaque somatic embryos (Figure 1).

RT-PCR

Indexing of 'Vero Beach' SE2 embryogenic cultures, somatic embryos and regenerated plants demonstrated that all material was ASBVd-infected. Agarose gel electrophoresis demonstrated that RT-PCR amplified RNA isolated from 'Vero Beach' SE2 embryogenic cultures and somatic embryos migrated between 200 and 300 bp, which was similar to the ASBVd-positive 'Lima Late' C4; and 'Vero Beach' SE2 floral tissue (Figure 2). There was no amplification with the negative control 'Hass' 11.4.3. Agarose gel electrophoresis also demonstrated that RT-PCR amplified RNA isolated from 'Vero Beach' SE2 plants migrated between 200 and 300 bp, which was similar to the ASBVd-positive 'Irwin', 'Yama', 'Vero Beach' 1 and floral tissue of 'Vero Beach' SE2 (Figure 3). There was no amplification of RNA isolated from ASBVd-free 'Hass' 11.4.3.

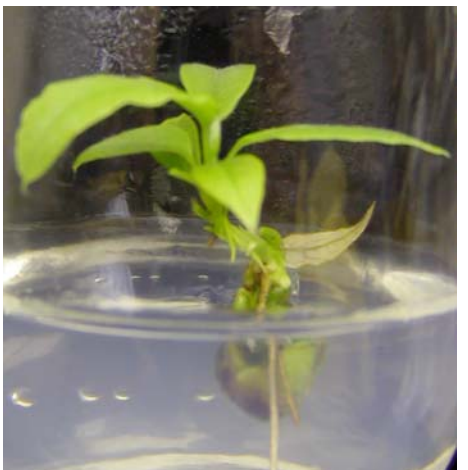

Figure 1. Avocado plant regeneration from embryogenic 'Vero Beach' SE2 nucellar culture.

Figure 2. Agarose gel of RT-PCR amplified products from embryogenic cultures. Lane 1 = 'Vero Beach' SE2 PEMs; Lane 2 = 'Vero Beach' SE2 somatic embryos; Lane 3 = 'Hass' 11.4.3 PEMs; Lane 4 = Clone 'Lima Late' C4; Line;5 = 'Vero Beach' SE2 floral tissue.

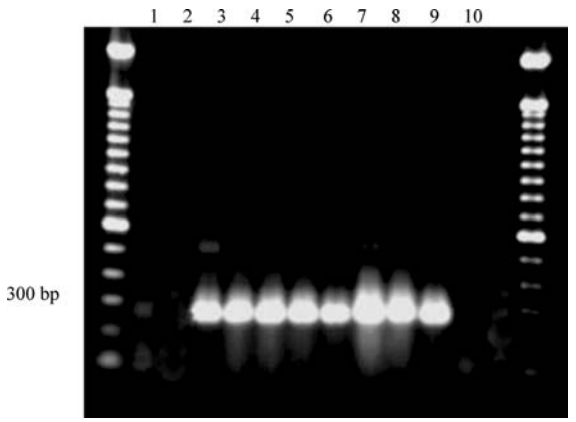

Figure 3. Agarose gel electrophoresis of RT-PCR amplified products. Lanes 1 and 10 = 'Hass' 11.4.3 PEMs; Lanes 2 to 4 = 'Irwin' PEMs, 'Yama' 381 PEMs and 'Vero Beach' 1 PEMs, respectively; Lane 5 = Somatic embryos of 'Vero Beach' SE2; Lanes 6 to 8 = Nucellar plants 1, 2 and 3, respectively; Lane 9 = Floral tissue of 'Vero Beach' SE2.

Cloning and sequencing

Tables 1 and 2 demonstrate the nucleotide changes that were detected in cDNAs from embryogenic cultures, somatic embryos and regenerated plants. There were 12 cDNAs recovered from PEMs; eight cDNAs from somatic embryos (>0.5 cm) and 79 cDNAs from the three regenerated plants (Tables 1 and 2). All clones contained sequences with ≥97% similarity to the reported ASBVd J02020 (Figure 4). The variants detected were the result of several indels and base exchanges, and some of them were present more than once in the variant population. Four of the 16 sequenced clones, 1 from PEMs and 3 from somatic embryos, were similar to the wild type variant J02020. In addition, there were four more ASBVd variants isolated from PEMs (B1, CF3, CF24 and CF62) and four additional isolates from somatic embryos (A2, CF56, CF63 and CF64).

A single variant isolated from three clones of PEMs had double U to A exchanges at nucleotides 3 and 5, respectively, and a U addition between nucleotides 230 and 236. One more variant isolated from two clones of PEMs had a U to A exchange at nucleotide 5 and an A deletion at nucleotide 7. A third variant isolated from PEMs had a U to A exchange at nucleotide 3, an A deleted at nucleotide 7 and an A addition between nucleotides 122 and 128. A single variant from PEMs had a U to A exchange at nucleotide 3, an A deleted at nucleotide 7 and a U addition between nucleotides 230 and 236.

Table 1. Sequence variants obtained from PEMs and somatic embryos of Vero Beach SE2 after 1 year

Sequence variant	Tissue	Clones	Residue in J02020	Nucleotide Change	Reference
J02020	PEM	4	–	–	Symons (1981)
B-1	PEM	3	3	U->A	Semancik and Szychowsky (1994)
			5	U->A	
			230–236	+U	
CF3	PEM	2	3	U->A	Schnell et al. (2001a, b)
			7	-A	
CF24	PEM	2	3	U->A	Schnell et al. (2001a, b)
			7	-A	
			122–128	+A	
CF62	PEM	1	3	U->A	No
			7	-A	
			230–236	+U	
A-2	SE	1	122–128	+A	Semancik and Szychowsky (1994)
CF56	SE	1	122–128	+AA	Schnell et al. (2001a, b)
J02020	SE	4	–	–	Symons (1981)
CF63	SE	1	122–128	+A	No
			16	A->G	
CF64	SE	1	72–73	+U	No
			213	C->U	

Table 2. Sequence variants of ASBVd from regenerated plants of 'Vero Beach' SE2

Sequence variant	Clones	Residue in J20202	Base change	Number of bases	Reported
Nucellus-recovered plant number 1					
J02020	4	-	-	247	Symons (1981)
CF68	2	C- > U	215	247	No
CF3	2	U- > A	3	246	Schnell et al. (2001a, b)
		-A	7		
CF24	1	U- > A	3	247	Schnell et al. (2001a, b)
		-A	7		
		+ A	122–128		
CF69	1	C- > U	213	247	No
CF70 5	1	AU- > UA	3–4	247	No
A-2	1	+ A	122–128	248	Semancik and Szychowsky (1994)
CF71	1	U- > C	150	247	No
		G- > A	186		
CF72	1	C- > G	155	247	No
CF73	1	G- > A	174	247	No
CF74	1	U- > A	3	246	No
		-A	7		
		U- > C	210		
Nucellus-recovered plant number 2					
CF3	3	U- > A	3	246	Schnell et al. (2001a, b)
		-A	7		
CF13	4	U- > A	3	247	Schnell et al. (2001a, b)
		U- > A	5		
CF24	2	U- > A	3	247	Schnell et al. (2001a, b)
		-A	7		
		+ A	122–128		
CF2	2	U- > A	3	247	Schnell et al. (2001a, b)
CF5	1	U- > A	3	246	Schnell et al. (2001a, b)
		-A	7		
		G- > A	207		
CF75	1	-A	7	246	
		G- > A	207		
CF76	1	U- > AA	3	248	No
		U- > A	5		
		G- > A	207		
CF77	2	-A	7	246	No
		U- > A	5		
		A- > G	131		
		G- > A	207		
CF 78	1	U- > A	3	248	No
		U- > C	181		
		+ A	122–128		
CF79	1	U- > A	3	248	No
		U- > A	5		
		+ U	129–130		
A-2	1	+ A	122–128	248	Semancik and Szychowsky (1994)

Table 2. (Continued)

Sequence variant	Clones	Residue in J20202	Base change	Number of bases	Reported
Nucellus-recovered plant number 1					
CF3	16	U- > A	3	246	Schnell et al. (2001a, b)
		-A	7		
CF12	4	U- > A	5	247	Schnell et al. (2001a, b)
CF2	4	U- > A	3	247	Schnell et al. (2001a, b)
CF80	2	UA- > AU	3–4	248	No
		+ A	122–128		
CF13	1	U- > A	3	247	Schnell et al. (2001a, b)
		U- > A	5		
CF8	2	U- > A	3	248	Schnell et al. (2001a, b)
		U- > A	5		
		+ A	122–128		
CF24	3	U- > A	3	247	Schnell et al. (2001a, b)
		-A	7		
		+ A	122–128		
CF81	2	U- > A	5	247	No
		U- > C	150		
CF82	2	U- > A	3	245	No
		-A	7		
		-U	115–118		
CF34	1	U- > A	3	248	Schnell et al. (2001a, b)
		-A	7		
		+ AA	122–128		
CF83	1	U- > A	3	246	No
		-A	7		
		C- > U	135		
CF84	1	U- > A	3	246	No
		-A	7		
		U- > C	26		
CF85	1	U > -A	3	245	No
		-A	7		
		-A	122–128		
CF86	1	U- > A	3	240	No
		-A	7		
		A- > G	179		
CF87	1	U- > A	3	246	
		-A	7		
		A- > G	240		
CF88	1	-U	3	248	No
		A- > U	7		
		+ AA	122–128		
CF89	1	U- > A	3	247	No
		U- > A	5		
		U- > C	217		

Variants isolated from somatic embryos were distributed as follows: one variant had an AA addition between nucleotides 122 and 128, a second variant presented a single A addition in the same region and a third variant had a single A addition between nucleotides 122 and 128 and an

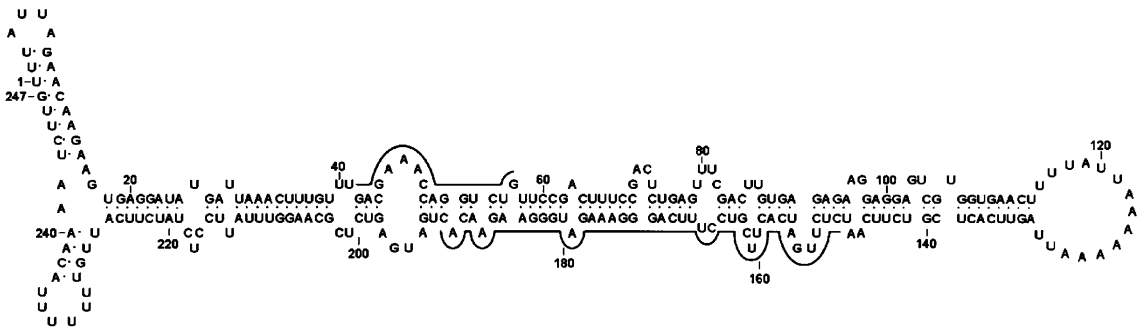

Figure 4. Sequence and secondary structure of ASBVd strain J02020 (Navarro and Flores, 2000).

A to G exchange at nucleotide 16. The only variant with changes in the stem area was isolated from somatic embryos and presented a U addition between nucleotides 72 and 73 and a C to U exchange at nucleotide 213.

Most of the variation involving ASBVd isolated from PEMs and somatic embryos occurred in the left and right terminal loops with only two variations observed in the stem area, indicating that variations occurred at specific sites rather than randomly (Table 3). The changes in the left terminal loop were detected at nucleotides 3, 5, 7, 16 and 230–236, while variations at the right terminal loop were exclusive to the 122–128 nucleotide region. The two changes observed in the stem area occurred at nucleotides 72–73 and 213.

Seventy-nine cDNAs harboring sequences with >97% similarity to ASBVd wild-type variant J02020 were isolated and sequenced from the 3 nucellar plants (Table 2): 16 from nucellar plant number 1 (NPT1), 19 from nucellar plant number 2 (NPT2) and 43 from nucellar plant number 3 (NPT3). The most common changes involved a U to A exchange at nucleotide 3 and occurred in 66 clones. A U to A change at nucleotide 5 was present in 24 clones. There was an A deletion at nucleotide 7 in 41 clones and an A or AA addition between nucleotides 122 and 128 was present in 19 sequenced clones. Fourteen changes in the stem area of the viroid molecule appeared in the three nucellar plants (Table 3). Out of the 14 changes observed in the viroid stem region of nucellar plants, only one (U to C at nucleotide 26) was observed in the upper region of the plus (+) strand. A reduction in the right terminal loop was detected in two sequenced clones of nucellar

Table 3. Distribution of the number of variants isolated from *in vitro* tissues with respect to nucleotide exchanges in the ASBVd molecule

Area in ASBVd molecule	Position	Exchange	PEMs	SE	NPT1	NPT2	NPT3
LTLs	3	U->A	4		4	8	15
	5	U->A	1			4	5
	7	-A	3		3	5	10
	230–236	+U	1				
RTL	122–128	+A	1	2	2	2	3
	122–128	+AA		1			2
	122–128	-A					1
	115–118	-U					1
	129	+U				1	
SR	16	A->G		1			
	72	+U		1			
	213	C->U		1	1		
	215	C->U			1		
	150	U->C			1		1
	186	G->A			1		
	165	C->G			1		
	174	G->A			1		
	210	U->C			1		
	207	G->A				4	
	131	A->G				1	
	181	U->C				1	
	135	C->U					1
	26	U->C					1
	179	A->G					1
	240	A->G					1
	217	U->C					1
	148	U->A					

(LTLs = Left Terminal Loops; RTL = Right Terminal Loop; SR = Stem Region).

plant 3; one had a U deletion between nucleotides 115 and 118, while another presented an A deletion in the 122–128 poly-A stretch. Twenty-two new sequences were isolated from the regenerated plants: CF68 to CF74 were isolated from NPT1, CF75 to CF79 were cloned from NPT2 and CF80 to CF89 were isolated from NPT3.

The ASBVd variants were grouped by cluster analysis into categories that could be defined on the basis of tissue, i.e., PEMs, somatic embryos and each of the regenerated plants (Figure 5).

Discussion

Avocado sunblotch viroid was not eliminated from infected clonal 'Vero Beach SE2' avocado by nucellar culture. Viroid elimination by nucellar culture from infected clonal citrus material has been reported with citrus exocortis viroid (*Pospiviroidae*) (Bitters et al., 1972). Another strategy for viroid elimination, which involves micrografting, has also been ineffective for eliminating ASBVd from infected avocado (Suarez et al., 2005). Therefore, standard protocols that have been utilized successfully for eliminating viroids from other species are ineffective for eliminating ASBVd from avocado.

ASBVd was persistent in embryogenic cultures, somatic embryos and regenerated plants. Maintenance of potato spindle tuber viroid (*Pospiviroidae*) in potato and tomato suspension cultures has been reported (Muhlbach and Sanders, 1981). Sequence variability of ASBVd was observed, particularly from regenerated plants. The observation that ASBVd sequence variants can develop in embryogenic cultures, somatic embryos and regenerated plants indicates that *in vitro* conditions favor ASBVd replication and variant evolution.

Nucleotide variation appeared to be related to the developmental stages of the cultures, and this was verified by cluster analysis. Three newly sequenced ASBVd variants were isolated from PEMs and somatic embryos. Only one of the six different position changes found in clones from PEMs occurred in the right terminal loop; whereas, five nucleotide changes were restricted to the left terminal region. Only a single variant isolated from somatic embryos had a nucleotide change localized in the left terminal region and

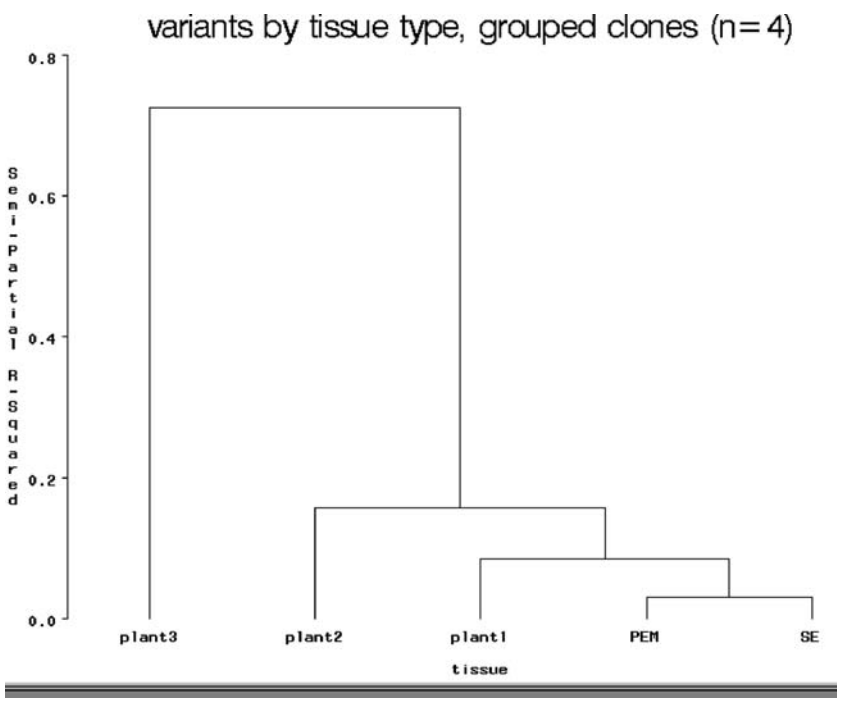

Figure 5. Cluster analysis of ASBVd variants that were identified in embryogenic cultures (PEM), somatic embryos (SE) and regenerated plants of avocado.

nucleotide changes in the right terminal loop were associated with sequences isolated from fully differentiated white-opaque somatic embryos. A U to A exchange at nucleotide 3 was present in all the sequence variants isolated from PEMs, (except J02020); however, this exchange was not found in any of the clones isolated from somatic embryos. Changes in the *in vitro* environment and/or differential gene expression during SED might therefore enhance ASBVd sequence variation.

Twenty-two newly described ASBVd sequences were isolated from three nucellar plants and most of these variants resulted from changes that occurred between nucleotides 131 and 226 in the stem region. Several of these variations occurred within the region of the ribozyme hammerhead formation; however it is unknown whether these changes affect the self-cleavage process of the ASBVd molecule.

ASBVd replication occurs within the chloroplasts, where the intermediate products of replication process are associated with the thylakoid membranes (Bonfliglioli et al., 1994). This would explain the large number of ASBVd variants that were isolated from regenerated plants in comparison with embryogenic cultures and somatic embryos. Chloroplasts are not present in avocado embryogenic cultures and in somatic embryos during their development. Proplastids are smaller than chloroplasts and lack thylakoid membranes. They are also in much lower copy numbers than chloroplasts, which accounts for lower levels of transcriptional gene activity (Baumgarner et al., 1989). Plastids contain both nuclear encoded and plastid encoded gene products (Martin et al., 1990; Abdallah et al., 2000). Although expression of nuclear encoded genes in the proplastids is possible (Harrak et al., 1995), high levels of transcription occur only during the proplastid to chloroplast conversion and in fully developed chloroplasts (Bisanz-Seyer et al., 1989; Baumgartner et al., 1993). Mutant genes can block proplastid to chloroplast conversion, ndicating that specific genes control this transition (Keddie et al., 1996; Chatterjee et al., 1996; Mandel et al., 1996). Studies with fungal and bacterial toxins that inhibit activity of nuclear and plastid polymerases have suggested that a nuclear encoded polymerase is most likely associated with ASBVd replication. Navarro and Flores (2000) reported that the initiation 5′ sites of both polarity strands of ASBVd are similar to the promoter region of two chloroplastic genes transcribed by a nuclear encoded polymerase. Daros and Flores (2002) isolated two proteins associated with ASBVd RNAs *in vivo* that contain transit peptide sequences capable of transporting products from the cytoplasm into the chloroplasts, facilitating the ASBVd hammerhead-mediated self-cleavage.

A relatively low number of ASBVd variants has been isolated from nonchloroplastic tissues despite the fact that embryogenic avocado cultures consist of several hundred or thousand PEMs, each of which can develop either as a single somatic embryo or as multiple somatic embryos (Witjaksono and Litz, 1999b). It is possible that a specific gene product involved in the proplastid to chloroplast conversion might trigger a high number of ASBVd variants. The data strongly suggest that a gene product involved in the proplastid to chloroplast conversion could be responsible not only for an increased ASBVd replication level but also for a higher rate of variation.

ASBVd was not eliminated from nucellar cultures. However, this study demonstrates that the embryogenic pathway is an important tool for exploring the biology of the viroid and the host-pathogen interaction. The embryogenic system could ultimately be utilized to evaluate strategies for ASBVd control at the cellular level.

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